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SYMBIOTIC ALGAE AND ^{14}C INCORPORATION IN THE FRESHWATER CLAM, *ANODONTA*

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Freshwater clams of the genus *Anodonta* have been reported to associate with zoochlorellae which are the symbiotic counterparts of the free-living *Chlorella* and which inhabit a variety of freshwater invertebrates. The first extensive discussion of the clam-algae association was by Goetsh and Scheuring (1926), who described the zoochlorellae as being distributed throughout the inner tissue layers of the posterior mantle, the posterior region of the gills, and the posterior foot. In other organisms, especially the green hydra, *Hydra viridis,* and *Paramecium bursaria,* zoochlorellae have been clearly shown to augment the host's growth (Muscatine and Lenhoff, 1965; Karakashian, 1963) via products produced by the algae and released to the host's tissues (reviewed in Smith *et al.*, 1969). In the present work the zoochlorellae associated with *Anodonta* are analyzed for their ability to photosynthesize and translocate products to the host *in vivo* and to the medium *in vitro*. The fate of translocated products in the host is examined. Data are presented regarding the degree of algal infection among individual clams of different size. The distribution of symbionts among the various infected tissues of individuals is also assessed.

Materials and Methods

Experimental organisms

Specimens of *Anodonta* (tentatively identified as *Anodonta grandis*) were collected during the months of July and August from Windy Pond, a freshwater reservoir located about 10 miles north of Woods Hole, Massachusetts. Following collection the animals were maintained at room temperature under ambient light in aerated tap water until used in experiments. Animals were also collected for the purpose of determining their wet weight and degree of algal infection. These determinations were done in the field and the animals returned to their habitat after analysis.

Algal symbionts were harvested from green specimens of *Anodonta* by homogenizing pre-dissected clam tissues in millepore filtered (0.4 μ) pond water (MFP) using a Waring blendor. The crude homogenate was filtered through five layers of cheesecloth and the resulting filtrate was further homogenized using a Dounce homogenizer. The algal cells were sedimented from this homogenate by low speed centrifugation ($500 \times g$) using a clinical centrifuge. The algal pellet was washed 3–5 times with MFP and finally resuspended in MFP for use in labelling experiments.

Incubation with $^{14}CO_2$

Whole animals were incubated for 3 hr in 300 ml of MFP containing 50 μCi $NaH^{14}CO_3$. During the incubation the animals were illuminated by a bank of six

15-watt fluorescent tubes which emitted 20 microeinsteins of photosynthetically active radiation (PAR) as measured by a Licor quantum sensor and counter. In dark experiments the animals were incubated in containers wrapped with aluminum foil.

Isolated algae (0.5 ml wet-packed) were incubated in 10 ml of MFP containing 10 μCi $NaH^{14}CO_3$. In some instances isolated algae were incubated in MFP buffered at pH7, 6.4, or 5.5, with McIlvane's citrate buffer. Algal suspensions were either illuminated by a bank of six 15-watt fluorescent tubes (20 microeinsteins PAR) or kept in the dark by wrapping the incubation vessels with aluminum foil. Incubations were carried out for $2\frac{1}{2}$ hr at room temperature after which the symbionts were centrifuged from the medium. Acidified samples of algae and medium were analyzed for ^{14}C by suspension in Aquasol and counting in a Beckman L.S. 100 scintillation spectrometer. Counting data were corrected for background and quench (quench never exceeded 10%).

Distribution of radioactivity in clam tissue

Following incubation in radioactive medium, animals were rinsed several times with MFP and dissected. Gills, mantle tissue, foot, and digestive gland were removed and homogenized separately in MFP using a Potter-Elvenhejem tissue homogenizer. The homogenates were fractionated after the method of Roberts *et al.* (1957) yielding a trichloroacetic acid (TCA) soluble fraction (mainly small molecules), an alcohol-soluble fraction (mainly lipids), a hot-TCA-soluble fraction (nucleic acids) and an alcohol- and TCA-insoluble fraction (proteins). In one experiment, blood collected by heart puncture was also fractionated. Samples of chemical fractions were transferred to scintillation vials containing Aquasol and counted in a Beckman L.S. 100 scintillation spectrometer. Counting data were corrected for background and sample quench.

Protein and chlorophyll determinations

The amounts of chlorophyll and protein contained in various clam tissues were determined by analyzing tissue discs. These discs were cut using a 1 cm cork borer and were homogenized in a tissue grinder following premincing with a razor blade. Algal cells were centrifuged from these homogenates, washed twice with MFP and finally extracted with absolute methanol for 24 hr at 5° C. Extracts were analyzed for total chlorophyll at 650 and 665 mμ by the method of MacKinney (1941) using a Bausch and Lomb Spectronic 20. The animal tissue homogenates were anlyzed for protein content by the Lowry *et al.* (1951) method using a standard curve prepared from partially purified *Anodonta* protein.

Results

Specimens of *Anodonta* were found at depths ranging from about 0.5–2 M. The clams lay superficially embedded in soft mud with the posterior margins of their valves just protruding above a fine layer of silt.

Not all clams exhibited an algal infection. As judged by superficial visual inspection, the degree of infection appeared correlated with the size of the clam: large clams ($\bar{x} = 72.03$ g $\pm$ s.d. 13.46, n = 14) exhibited heavy infection, especially of the gills and mantle; small specimens ($\bar{x} = 40.20$ g $\pm$ s.d. 19.70, n = 16) exhibited no external evidence of infection. The larger specimens exhibited valves

TABLE I

Amount of chlorophyll and protein in 1-cm tissue discs taken from various regions of Anodonta *specimens. Data expressed as $\bar{x} \pm$ s.d., $n = 3$ discs for each determination.*

	Chlorophyll (μg)	Protein (μg $\times 10^{-3}$)	Chlorophyll/Protein $\times 10^{-4}$
posterior gill	1.67±0.37	5.22±1.20	3.2
mid gill	1.13±1.20	4.58±2.50	2.5
anterior gill	1.10±1.50	3.97±1.25	2.8
mid mantle	6.53±2.95	6.98±2.45	9.4
posterior mantle	0.52±0.49	1.73±1.19	3.0

that were pocked, abraded, and on occasion completely perforated, thus exposing the underlying mantle.

When clams were dissected it was observed that the green symbionts were more densely aggregated towards the posterior end of the animal. The folded, leaf-like organization of the mantle and gills lent itself to the preparation of tissue discs cut from these organs. Analysis of these discs for chlorophyll (originating mainly from zoochlorellae) gave an indirect estimate of the relative distribution of symbionts within the gills and mantle. Table I lists the chlorophyll content, protein content, and chlorophyll : protein ratio of discs cut from the gills and mantle of large (60–80 g) clams. From Table I it can be seen that the greatest amount of chlorophyll and by inference the greatest number of symbionts were detected in the mid-mantle region. This region was also the greenest. The lowest cholorphyll per disc was measured in the posterior mantle (Table I). This region of the mantle appeared darkly pigmented, obscuring the symbionts and making it impossible to judge the degree of infection by eye. The chlorophyll : protein ratio was fairly constant among the discs from the various regions (≈ 0.0003, Table I) with the exception of the mid-mantle region, which exhibited a ratio of 0.00094 (Table I), reflecting the greater amount of chlorophyll in this region.

The data in Table II show that the total incorporation of ^{14}C by animals incubated in the light (average of experiments I and II) is approximately seven times the total incorporated by animals in the dark (average of experiments III and IV). Further, Table III shows the distribution of ^{14}C among the various chemical fractions prepared from tissues of clams incubated in either light or dark. The largest amount of ^{14}C incorporated occurs in the TCA-soluble fractions from clams incubated in the light. Among the tissues analyzed, the gills and blood showed the greatest amount of ^{14}C in this fraction. This fraction also contained the highest percentage of ^{14}C (75–95%) of all fractions irrespective of the tissue origin. By contrast, the TCA-soluble fractions from tissues of dark-incubated clams contained approximaely $\frac{1}{10}$ to $\frac{1}{5}$ as much ^{14}C as their illuminated counterparts. The distribution of the label in these fractions differed strikingly from that observed in the light experiments. Whereas the TCA-soluble fraction invariably exhibited the highest percentages of ^{14}C (75–95%) in illuminated clams, it contained lower percentages (27–67%) in the dark-incubated clams. There was a concomitant increase in the percentages of ^{14}C in the alcohol-soluble and hot-TCA-soluble fractions from dark-incubated clams, amounting to some 10–40%. By comparison, with the exception of the mantle tissue of experiment I, the per cent of ^{14}C in the alcohol-soluble and hot-TCA-soluble fractions of experiments I and II never exceeded 9%. Finally it can be seen from Table II that the total ^{14}C measured

TABLE II

Distribution of radioactivity in different fractions of clam tissue in four experiments. For each entry the first figure is the percent ^{14}C in that fraction; the second figure is the total ^{14}C expressed as dpm/mg clam protein in the initial tissue homogenate.

	TCA sol.	Alc. sol.	Hot TCA sol.	Alc. insol., Hot TCA insol.
Incubated in Light				
Experiment I				
Digestive gland	87.6, 3,490	5.6, 222	4.8, 192	2.2, 86
Foot	91.3, 2,453	1.1, 29	4.6, 124	3.0, 81
Gills	82.3, 9,529	4.1, 477	8.3, 961	5.3, 611
Mantle	75.7, 2,882	4.8, 184	17.8, 654	2.3, 86
Total (all fractions and tissues) = 22,061 dpm/mg protein				
Experiment II				
Digestive gland	89.3, 1,072	4.2, 51	3.6, 44	2.8, 34
Foot	88.2, 2,369	6.4, 172	2.3, 64	2.9, 80
Gills	79.1, 3,658	7.3, 339	7.5, 349	6.0, 278
Mantle	82.8, 3,261	6.8, 269	7.7, 305	2.6, 101
Blood	95.4, 20,384	2.5, 536	1.4, 300	0.6, 138
Total (all fractions and tissues) = 33,804 dpm/mg protein				
Incubated in Dark				
Experiment III				
Digestive gland	27.2, 490	20.8, 375	43.9, 792	8.1, 146
Foot	41.7, 220	29.8, 157	21.6, 114	6.8, 36
Gills	60.9, 331	11.0, 56	22.9, 117	5.3, 27
Mantle	32.2, 306	10.3, 123	39.5, 473	18.0, 216
Total (all fractions and tissues) = 3,959 dpm/mg protein				
Experiment IV				
Digestive gland	48.4, 531	12.8, 141	35.4, 390	3.3, 36
Foot	37.1, 117	33.9, 107	24.8, 78	4.1, 13
Gills	54.8, 201	16.2, 60	21.8, 80	7.1, 26
Mantle	66.9, 269	14.7, 59	15.7, 63	3.7, 11
Blood	44.7, 678	41.8, 634	6.5, 98	7.1, 108
Total (all fractions and tissues) = 3,700 dpm/mg protein				

in the alcohol-soluble, hot-TCA-soluble, and alcohol-insoluble, hot-TCA-insoluble fractions ranged from 11 dpms to 961 dpms in the light experiments and 27 dpms to 490 dpms in the dark experiment. In all experiments the alcohol-insoluble, hot-TCA-insoluble fractions contained the lowest activity and the lowest percentage of the ^{14}C incorporated.

TABLE III

Effect of pH on translocation of ^{14}C in vitro *by symbionts harvested from* Anodonta *specimens*

pH	Total ^{14}C fixed (dpm)	Percent of ^{14}C in medium
7.4	1,939,655	10.8
6.5	1,074,935	16.6
5.5	828,455	19.6

Table III shows the results of *in vitro* experiments exposing isolated symbionts to $NaH^{14}CO_3$ in MFP adjusted to three different pH's. Analysis of acidified samples showed a significant percentage of acid-precipitable ^{14}C in the medium at pH 7.4. This percentage nearly doubled at pH 5.5. Total ^{14}C fixed (symbionts + medium) decreased with decreasing pH.

Discussion

The degree of algal infection of individual clams appears correlated with the size/age of the clams. Large (older) clams exhibit the greatest degree of infection. While the details of infection are not known, the data suggest that infection may be a progressive process beginning, perhaps, in the clam's early adulthood. Individual clams might acquire symbionts from the environment and these could multiply slowly within the host's tissues. Alternatively, infection with zoochlorellae could be continuous, with symbionts constantly being derived from the environment and added to the standing crop in the clam.

Valves of the largest clams were often chipped and pitted, giving rise to a thinner and more translucent shell material in the central portion of the valve. On occasion there were intense green areas on the tissues underlying these "windows." Examination of tissue from these regions revealed a high density of zoochlorellae. These observations suggest that zoochlorellae tend to congregate where there is more light, perhaps as a result of enhanced cell division. Goetch and Scheuring (1926) attributed this pitting and chipping of the valves to effects of the parasitic [sic] algae. Examination of valves from specimens of *Anodonta* collected at Windy Pond revealed that the pitting took place inward from the outer surface, suggesting some external environmental cause of degradation of the shell material.

The major symbiont-bearing tissues of the clams were the gills and mantle. These were fairly uniform with respect to the distribution of chlorophyll (Table I) with the exception of the mid-mantle. This region exhibited over three times the chlorophyll per unit protein of any other region sampled. This part of the mantle, which invariably lay under the chipped and pitted portion of the clam's valves, looked greener than other parts. Occasionally aggregations of symbionts were observed in the superficial tissue of the foot.

Data from four experiments, in Table II, strongly suggest the viability and photosynthetic capacity of the zoochlorellae associated with *Anodonta* tissue. Incorporation of ^{14}C by clams illuminated in the light averages seven times greater than dark incorporation. Fixation of ^{14}C by clam tissues is not insignificant. However, in terms of respiratory biomass, the clam tissue undoubtedly exceeds the zoochlorellae by several orders of magnitude. Hence the apparent large dark-fixation component of the total ^{14}C fixed. By comparison, light fixation of ^{14}C in hydra is ten times greater than dark incorporation (R. Pardy, unpublished data), and the respiratory biomass of the host in the hydra association is less than in the clam.

Radioactivity from ^{14}C is found in all chemical fractions of the host (Table II) with the largest proportion occurring in the small-molecule fractions of tissues from illuminated clams. The appearance of ^{14}C in the nucleic acid and protein fractions indicates that some ^{14}C, probably as small-molecule precursors, is metabolized into macromolecules. By comparison, ^{14}C from fractions of tissues from dark-incubated clams is generally more evenly distributed among the various chemical fractions, with the exception of the alcohol-insoluble, hot-TCA-insoluble fraction, which generally contained less. The digestive gland (experiments III and IV)

and mantle (experiment III) showed a comparatively high percentage of ^{14}C in the nucleic acid fraction. The reason for this is not clear: either these tissues metabolize the products of dark fixation differently or the results reflect experimental variation in the organisms or analytical techniques.

The ultimate fate of the large fraction of TCA-soluble molecules in the illuminated clams is only suggested by the data in Table II. Clearly some may be metabolized into lipids and macromolecules. However, the activity of these fractions is not appreciably higher than that observed in the corresponding fractions from the dark-incubated animals. Either the small molecules are rapidly respired or the duration of the experiments was too short to observe significant metabolism of these molecules into the other biochemical components. In the green hydra, Roffman & Lenhoff (1969) showed progressive movement of ^{14}C from the TCA-soluble fraction to the other fractions over several hours. Long-term experiments on *Anodonta* were not performed during the present investigation.

In other symbioses involving zoochlorellae, the source of ^{14}C in the host tissues is translocated metabolites that originate from symbiont photosynthesis (Muscatine, 1965; Muscatine *et al.*, 1967). Invariably, significant amounts of labelled organic material can be found in the medium when these symbionts are incubated *in vitro*. A similar finding has been obtained from *Anodonta* zoochlorellae (Table III). Depending upon the pH of the incubation medium, isolated symbionts contribute up to nearly 20% of their total fixed ^{14}C to the medium as acid-stable (organic) carbon. This argues for the existence of a translocating system that operates when the symbionts are *in situ* as well, providing a basis for photosynthetically fixed ^{14}C to enter the host's metabolic network. Table III also shows that the percentage of ^{14}C in the medium increases with decreasing pH. A similar dependency of translocation on pH has been shown for green-hydra symbionts (Muscatine, 1965; Cernichiari *et al.*, 1969). Hence it seems that the *Anodonta* symbionts may be related, at least functionally, to those zoochlorellae that inhabit hydra. The apparent decrease in total incorporation of ^{14}C with decreasing pH (Table III) might result from either an inhibition of photosynthesis and/or decreased availability of $NaH^{14}CO_3$.

The overall role of zoochlorellae in augmenting the metabolic and respiratory demands of *Anodonta* is not known. The products of translocation have not been identified. Moreover, the cytological and cellular aspects of this symbiosis have not been investigated adequately to define the spatial relationships between host and symbiont. Clearly, further research at both levels is needed before statements regarding the efficacy of the symbiosis and role of the symbionts can be made.

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Summary

1. Specimens of a freshwater clam *Anodonta* were found to be symbiotized with zoochlorellae. Larger, hence older, clams showed a greater degree of infection with algae.

2. In the light, clams exposed to the precursor $NaH^{14}CO_3$ showed a greater incorporation of ^{14}C than corresponding control animals incubated in the dark.

The difference is thought to result from the photosynthetic activities of the zoochlorellae.

3. Chemical fractionation of selected host tissues revealed that in the light, ^{14}C appeared in greatest amount in the small molecule fraction though it was also detected in other fractions.

4. The major source of ^{14}C in the hosts incubated in light is thought to be photosynthetic products translocated by the zoochlorellae. This view is strengthened by data showing that the symbionts can translocate to the medium when the algae are incubated *in vitro*.

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